

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/045, 31/12, 31/44, 31/335, 31/13	A2	(11) International Publication Number: WO 96/35415 (43) International Publication Date: 14 November 1996 (14.11.96)
(21) International Application Number: PCT/GB96/01118 (22) International Filing Date: 9 May 1996 (09.05.96) (30) Priority Data: 9509321.7 9 May 1995 (09.05.95) GB (71) Applicant (for all designated States except US): ZYLEPSIS LIMITED [GB/GB]; 6 Highpoint, Henwood Business Estate, Ashford, Kent TN24 8DH (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SIME, John, Thomas [GB/GB]; 31A Canon Woods Way, Kennington, Ashford, Kent TN24 9QY (GB). CHEETHAM, Peter, Samuel, James [GB/GB]; 35 Rochester Avenue, Canterbury, Kent CT1 3YE (GB). GRADLEY, Michelle, Lorraine [GB/GB]; 2 Somner Close, Canterbury, Kent CT2 8LJ (GB). BANISTER, Nigel, Eric [GB/GB]; 54 Pound Lane, Canterbury, Kent CT1 2BZ (GB). (74) Agent: BASSETT, Richard; Eric Potter Clarkson, St. Mary's Court, St. Mary's Gate, Nottingham NG1 1LE (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: USE OF SESQUITERPENES FOR INHIBITING OXIDATIVE ENZYMES (57) Abstract The invention relates to the use of a sesquiterpene for inhibiting an oxidative enzyme, preferably a cytochrome P450 enzyme. The invention also relates to compositions comprising a sesquiterpene and a drug which exhibits instability in the presence of an oxidative enzyme.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

USE OF SESQUITERPENES FOR INHIBITING OXIDATIVE ENZYMES

The present invention relates to substances and methods for inhibiting
5 oxidative enzymes, and in particular for inhibiting enzymes responsible for
the metabolism of bioactive agents to inactive or excretable derivatives
thereof *in vivo*.

Metabolism of bioactive agents (hereafter "drugs") to inactive or
10 excretable derivatives is undesirable in many treatment regimes because
it leads to a reduction in bioavailability. This may necessitate increasing
dosage of the drug and this can lead to adverse side effects. The most
significant enzymes responsible for such metabolism are the cytochrome
P450 enzymes such as the CYP3A4 isozyme. Most agents known to
15 inhibit CYP3A4 have significant adverse effects that make them unsuitable
for co-administration with the drug.

The present invention aims to overcome these and other problems of the
prior art.

20

The effect of grapefruit juice on the bioavailability of some drugs has been
reported recently. [Soons *et al*, Clin. Pharmacol. Ther., 1991, 50, 394;
Edgar *et al*, Eur. J. Clin. Pharmacol., (1992) 42, 313-317; and J. Clin.
Pharmacol., 1994, 46, 175 - for the hydroxylation of coumarin (CAS No
25 91-64-5)]. For example, Bailey *et al*, (1994) Clin. Pharmacokinet., 26(2),
91-98 showed that concomitant administration of a glass of regular
strength grapefruit juice (200 ml) more than doubled the mean maximal
plasma concentration (C_{max}) and area under the curve plasma concentration
- time curve (AUC) of Felodipine, a dihydropyridine calcium antagonist
30 for the standard (non-controlled release) tablet compared with C_{max} and

AUC values after administration of the drug with water. Because first-pass (pre-systemic) metabolism in the small intestinal wall and liver is the reason for an average bioavailability of felodipine of 15% after oral administration with water, it is likely that grapefruit juice inhibits first-pass metabolism. Similar effects were shown for other dihydropyridine calcium antagonists.

It has been asserted that the ingredient in grapefruit juice responsible for the above inhibitory effect is a bioflavonoid (Yee *et al.*, The Lancet, 1995, 345, 955; Br. J. Clin. Pharmac., 1993, 36, 457) and probably naringin which is present at a concentration of approximately 90 mg/200 ml of grapefruit juice. However, compared with grapefruit juice, naringin solution produced less interaction with felodipine in all volunteers tested by Bailey *et al* (1994).

15

The sesquiterpene compound, nootkatone, is one of the primary contributors to the distinctive flavour and aroma of grapefruit. Nootkatone expression in the fruit is linked to the maturity of the fruit and is associated with the maturation process.

20

In addition to plant produced nootkatone a number of enzyme systems produced by organisms other than higher plants have the capability of converting synthetic precursors of nootkatone to nootkatone itself. Thus, for example, a variety of microorganisms can oxidise valencene to nootkatone.

25

Whilst investigating such transformations as effected by bacteria and fungi, it was deduced that the conversion was the result of two oxidative steps. The first was indicated to be carried out by an enzyme thought to be a cytochrome P450 or related enzyme system. The second appeared

30

as an oxidoreductase transformation.

During elucidation of these enzymic steps it was, unexpectedly, discovered that nootkatone acts as an inhibitor of the first enzyme catalysed oxidative
5 step. This surprising effect was observed in many organisms, both eukaryotes and prokaryotes. Nootkatone was demonstrated to have use as an inhibitor of these enzyme systems *in vivo*.

The inventors have shown that their discovery of this inhibitory property
10 of sesquiterpenes, particularly oxidised sesquiterpenes such as nootkatol and nootkatone or isomers of nootkatone eg. α -vetivone can be extended to enzymes responsible for drug metabolism *in vivo* in mammalian systems. In particular, the effect of cytochrome P450 enzymes and related enzyme systems which process drugs and xenobiotics in mammals can be
15 modified.

According to the invention there is provided a sesquiterpene for use as a medicament.

20 In a second aspect of the invention provides a sesquiterpene for use in the inhibition of an oxidative enzyme.

The inhibition of an oxidative enzyme can be readily determined by a skilled person in an *in vitro* test in which the consumption of oxygen by
25 the oxidative enzyme is measured in the presence or absence of a sesquiterpene of the invention. The total inhibition or reduction of oxygen consumption in the presence of a sesquiterpene is indicative of an inhibitory effect of the invention.

30 In a third aspect the invention provides the use of a sesquiterpene in the

manufacture of a composition for stabilising a drug compound *in vivo*, or *in vitro*, wherein the drug compound exhibits instability in the presence of one or more oxidative enzymes.

- 5 In a fourth aspect the invention provides a selection method comprising screening a test sesquiterpene compound for an inhibitory effect on the oxidative activity of an enzyme and, if the test compound displays an inhibitory effect, using the compound in the manufacture of a medicament.
- 10 By "inhibitory" we include the meaning that the sesquiterpene reduces the extent of metabolism of the drug *in vitro* at a concentration of 500 μ g/ml and preferably 100 μ g/ml or less.

Preferably, the inhibitory effect is screened by measuring the
15 hydroxylation of the steroid 4-androstene-3,17-dione by the oxidative enzyme system of *Bacillus megaterium* (ATCC 13368).

Alternatively, the inhibitory effect can be screened by measuring the hydroxylation of valencene to nootkatol, using the methods described in
20 Japanese patent 06303-967/1991 or Chemiker-Zeitung, 1991, 115, 358; the disclosures of which are incorporated herein by reference.

The terpenes are a class of natural products having a structural relationship to isoprene

25

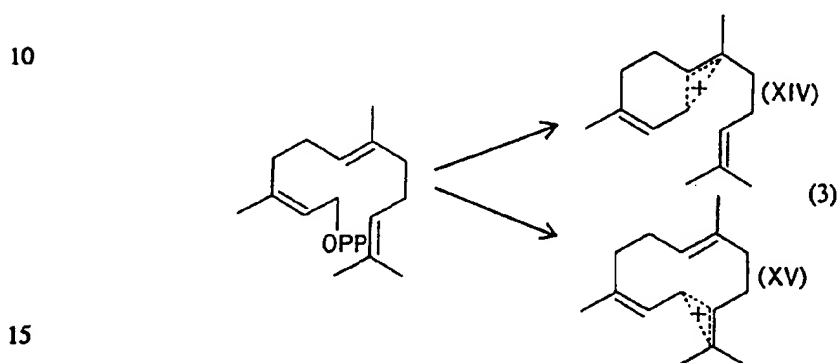


(1a)

30

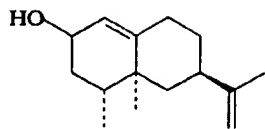
Terpenes are classified according to the number of isoprene units of which they are composed.

Sesquiterpenes are C_{15} hydrocarbons or their oxygenated analogs. They arise from the cyclization of farnesylpyrophosphate and subsequent rearrangements of the resulting carbonium ions (XIV and XV), as in reaction (3), where OPP represents oxygen-alkylated pyrophosphate. Almost all known sesquiterpenes can be derived from these two cations.

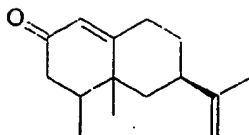


Many sesquiterpenes are important constituents of the characteristic aromas of plant products.

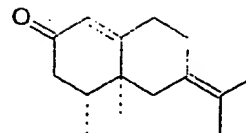
20 The preferred sesquiterpenes of the present invention are preferably substituted and advantageously bicyclic. The most preferred bicyclic sesquiterpenes are oxidised. Advantageously, the oxidised sesquiterpenes are the products of oxidation of valencene, nootkatol and more preferably nootkatone or isomers of nootkatone such as α -vetivone (isonootkatone).



nootkatol



nootkatone

 α -vetivone

For isolation and structure of α -vetivone see Pfau. Plattner, *Helv. Chim. Acta.*, **22**, 640 (1939); Naves. Perrottet, *ibid*, **24**, 3 (1991).

In a fifth aspect the invention provides a pharmaceutical composition comprising a sesquiterpene and one or more pharmaceutically acceptable carrier(s).

10

In a sixth aspect the invention provides a pharmaceutical composition comprising a sesquiterpene of the invention together with a drug which exhibits instability in the presence of an oxidative enzyme and one or more pharmaceutically acceptable carrier(s). The carriers must be "acceptable" in the sense of being compatible with the sesquiterpene and drug of the invention and not deleterious to the recipients thereof.

15

This aspect of the invention affords the advantage that the duration of the drug effect can be prolonged enough to reduce the frequency of daily dosage which is likely to improve patient compliance with the treatment regimen.

20

Preferably, the drug is a dihydropyridine calcium antagonist, especially those which are initially oxidised to their dehydro-derivative by cytochrome P450 3A4. Examples include nifedipine, felodipine, nitrendipene, nisoldipine, amlodipine, isradipine, nicardipine, nilvadipine

25

and nimodipine. For example nitrendipine, a chiral 1,4-dihydropyridine calcium entry blocker, is currently used in the treatment of hypertension. The systemic bioavailability of racemic nitrendipine is low and variable (5% to 30%) despite its almost complete gastrointestinal absorption. This
5 is caused by extensive presystemic biotransformation to the pharmacologically inactive primary pyridine metabolite dehydronitrendipine (M-1) by enzymes of the cytochrome P-450 IIIA family. The inhibitory sesquiterpenes of the invention display a particularly pronounced inhibitory effect in relation to drugs such as
10 nitrendipine which undergo a high degree of first-pass metabolism by cytochrome P450 oxidases.

A particularly preferred drug is cyclosporin. Cyclosporin is a cyclic undecapeptide of fungal origin which forms the cornerstone of therapy for
15 maintenance immunosuppression following transplantation. However, blood concentrations of cyclosporin must be maintained within a relatively narrow range (approximately 100-300 ng ml⁻¹) in order to achieve effective immunosuppression with a minimum of nephrotoxicity. This can be a difficult task due, in large part, to the wide inter-patient variability
20 in oral bioavailability ranging from less than 5% to as much as 90%.

Cyclosporin treatments regimes typically cost between 6,800-7,000 US dollars per annum.

25 Recent studies have suggested, that the poor and variable bioavailability of cyclosporin may relate to pre-systemic metabolism of cyclosporin in the gut wall. The major cyclosporin-metabolizing enzyme appears to be CYP3A4, a cytochrome P-450 enzyme present in high concentrations in the gut wall.

- As observed by Ducharme *et al*, (1993) Br. J. Clin. Pharmac., 36, 457, a compound which inhibits the pre-systemic metabolism of cyclosporin without systemic effects would be of great clinical value. The compound would improve bioavailability and decrease the cost of therapy through dose reduction in some patients. However, agents known to inhibit CYP3A4 such as erythromycin, verapamil, diltiazem and ketoconazole have significant adverse effects that make them unsuitable for co-administration with cyclosporin.
- Another preferred drug is the antineoplastic agent Taxol® which can be isolated from the bark of the Pacific yew tree, *Taxus brevifolia*. For isolation and structure of Taxol see M.C. Wani *et al.*, *J. Am. Chem. Soc.*, 93, 2325 (1971). For synthesis and anticancer activity of taxol derivatives see D.G.I. Kingston *et al*, *Studies in Organic Chemistry*, vol 26 entitled "New trends in natural products chemistry 1986", Atta-ur-Rahman, P.W. Le Quesne, Eds. (Elsevier, Amsterdam, 1986) pp 219-235.

Taxol® is available commercially from Bristol Myers Squibb.

- The preferred oxidised sesquiterpene of the invention, nootkatone, is present in grapefruit juice but not orange juice (which does not have an inhibitory effect on cytochrome P450 enzyme mediated drug metabolism). Hence, the inventors believe that nootkatone is the inhibitory component of grapefruit juice.

25

- As nootkatone is present in natural grapefruit juice it does not appear to be toxic, at least at the concentrations found in normal strength grapefruit juice (approximately 7mg/200ml). Thus, the sesquiterpenes of the invention have a clear advantage over known cytochrome P450 enzyme inhibitors which exhibit adverse effects on co-administration with a drug.
- 30

Clearly, the unexpected identification of the active inhibitory ingredient in grapefruit juice as a sesquiterpene is of clinical importance, because, due to the variability in the amount of active ingredients between natural grapefruit juice samples, it was impossible previously to quantify an effective inhibitory dose.

The inhibitory sesquiterpenes of the invention can be used with drugs having incomplete first-pass metabolism to produce a more consistent and predictable clinical response. This is particularly useful for drugs with steep concentration-response curves, narrow therapeutic ranges, toxic metabolites or nonlinear pharmacokinetics.

The sesquiterpenes of the invention are especially useful in individuals exhibiting an extensive metaboliser phenotype, identifiable by the genetic test disclosed in UK Patent No. 2,256,271 B.

Preferably, the composition is adapted for oral administration.

Currently, drugs that undergo complete first-pass metabolism are only administered by the intravenous route. The inhibitory sesquiterpenes of the invention permit such drugs to be administered orally, which is more tolerable for patients and better suited to long term treatment.

The compositions of the invention may be in the form of a solid which can be formed for example as tablets, capsules (including spansules) etc. Suitable pharmaceutically acceptable solid carriers include starch, lactose, dextrin and magnesium stearate.

The composition can also be in the form of a liquid, preferably a grapefruit juice flavoured drink. Preferred liquid carriers should be sterile

and pyrogen free. Examples are saline and water.

The compositions of the invention can also be adapted for parenteral administration and preferably intravenous administration. Inactivation of drugs in circulating body plasma by oxidative metabolic processes, often associated with the liver, is a well recognised phenomenon. Intravenous co-administration of an inhibitor of cytochrome P450 enzymes offers the potential to reduce this inactivation and so prolong the effective circulating concentration of bioactive compound.

10

It will be appreciated that the effective ratio of the amount of inhibitory sesquiterpene to the amount of drug will vary depending on the particular drug and its pharmacokinetic characteristics. A skilled worker can readily determine by routine tests an effective ratio of sesquiterpene to drug for use according to the invention. If the drug is cyclosporin for example, the prescribed cyclosporin dose may range from 150 to 350 mg day⁻¹. Preferably the amount of sesquiterpene such as the sesquiterpene nootkatone included in the formulation ranges from 7 to 16 mg.

Preferably, the sesquiterpene is present in an amount of from approximately 0.1 to 10 wt % especially 4 to 7 wt% and most preferably 5 wt% based on the amount of the drug in the composition. However, in view of the low toxicity of the sesquiterpenes of the invention it is expected that much higher amounts relative to the amount of drug can be administered without adverse effects.

Treatment with compositions of the invention may consist of a single dose or a plurality of doses over a period of time. The compositions of the invention may be formulated in unit dosage forms, ie. in the form of discrete portions containing a unit dose, or a multiple or sub-unit of a unit

30

dose.

In a sixth aspect the invention provides a product comprising a sesquiterpene and a drug compound which exhibits instability in the presence of an oxidative enzyme as a preparation for simultaneous, separate or sequential use in therapy. Preferably the sesquiterpene is nootkatone, α -vetivone or nootkatol. The drug is preferably cyclosporin, taxol or a dihydropyridine.

In a seventh aspect the invention provides a method of *in vitro* inhibition of an oxidative enzyme, which method comprises mixing the oxidative enzyme with a substituted or unsubstituted sesquiterpene. Preferably, the sesquiterpene is nootkatone, nootkatol or α -vetivone. Preferably the enzyme is a cytochrome P450 enzyme.

Preferred non-limiting embodiments of the invention will now be described with reference to the accompanying figures in which:

Figure 1 shows the effect of the oxidised sesquiterpene nootkatone on the formation of metabolites of the anticancer drug Taxol.

Example 1 - Measurements of cytochrome P450 enzyme inhibition

Solvents and reagents

- 5 ^3H -Cyclosporin A was purchased from Amersham. All other reagents were obtained from BDH (Poole, Dorset, UK) and were of Analar or HPLC grade. Bacto-agar, peptone and yeast extract were purchased from Difco. Glass beads (0.45-0.5mm) were purchased from Braun Melsungen AG. Thin layer chromatography (TLC) was conducted on Merck
10 (Germany) Silica gel 60 F₂₅₄ size 20x20 cm plastic sheets.

Microorganisms, growth conditions and spectral studies

- Human cytochrome P450 3A4 was expressed using the vector pAAH5
15 (p3A4) in *Saccharomyces cerevisiae* strain AH22 (Guengerich *et al.*, 1991). Plasmid DNA was prepared using Promega's Wizard kit and *S. cerevisiae* strain AH22 (*a his4-519 leu2-3,2-112*) was used for transformation. Transformants were grown and maintained at 30°C on minimal medium containing 2% (w/v) glucose, 0.67% (w/v) Difco yeast
20 nitrogen base without amino acids and 20 µg/ml histidine. Cytochrome P450 content and substrate binding studies were carried out spectrally using a Philips PU8800 US/Vis scanning spectrophotometer according to Omura and Sato (1964) and Jefcoate (1979) respectively. Protein content was determined by the method of Lowry *et al.* 1956. Yeast microsomal
25 fractions used in reduced carbon monoxide difference spectra, substrate binding studies and metabolism studies were prepared as described previously (Mehmood *et al.*, 1995). This involved mechanical breakage of the yeast cells (harvested at 5×10^7 cells/ml) using a Braun MSK homogeniser and centrifugation to pellet cells and mitochondria prior to
30 harvesting microsomes after 1h, 100000g. Microsomal fractions were

resuspended using buffer containing 20% (v/v) glycerol 0.2 M potassium phosphate, 1 mM EDTA pH 7.4 to a final protein concentration of 2 mg/ml. For measurement of cytochrome P450 a few grains of sodium dithionite were added and the sample divided between two cuvettes. CO
5 was bubbled into the sample cuvette and the difference spectrum was recorded between 390-500 nm. Substrate binding spectra were obtained by recording difference spectra between 350 and 500 nm using split-cell cuvettes. The cuvettes contain microsomal fractions or buffer in each chamber of the sample and reference split cuvettes. Nootkatone was
10 added to the microsomal fractions in the sample cuvette but to the buffer in the reference cuvette and difference spectra recorded between 350-500 nm.

In vitro biotransformation

15 Microsomal samples containing 100 pmoles P450 3A4 and an equivalent concentration of microsomal protein from control yeast containing pAAH5 were preincubated with cyclosporin A (110pmol; 1 μ Ci), at 37°C in buffer containing 20% (v/v) glycerol, 0.2 M potassium phosphate, 1 mM EDTA
20 pH 7.4. The reaction was initiated by the addition of 1 mM NADPH in a final volume of 1 ml and after 30 min. terminated by adding 1 ml ethyl acetate in each reaction mixture. Control experiments confirming the involvement of P450 3A4 in the biotransformation involved the use of microsomes from transformants carrying pAAH5 alone, bubbling carbon
25 monoxide through the reaction for 30 seconds prior to adding substrate and NADPH and lastly the omission of NADPH from reactions. Nootkatone dissolved in DMSO was added at various concentrations to establish inhibitory effects. Microsomal fractions were extracted three times by the addition of 1 ml hexane, vortexing for 20 seconds and
30 separating layers by centrifugation (3000 rev min⁻¹) for 3 min. The

organic layer was taken and the residual water was removed by adding a few milligrams of anhydrous sodium sulphate. Hexane extracts were dried under a stream of nitrogen at 45°C in a water bath and resuspended in hexane again for TLC analysis.

5

Thin layer chromatography

The ethyl acetate extracts from biotransformation studies were directly spotted on to TLC plates and developed in solvent system
10 heptane/pyridine/ethyl acetate (100/75/1 v/v) in a chromatographic tank lined with a blotting paper the same size as the TLC plate. Chromatograms were air-dried and visualised under UV light or in iodine vapours. The R_f values of spots were measured and compared to the literature. Three metabolites were identified (M1, M6 and M21) and
15 spots corresponding to substrate and metabolic were cut from the chromatogram and eluted twice in 1 ml ethyl acetate. Radioactivity measurements by scintillation counting were used to determine the extent of metabolism.

20 Guengerich F P, Brian W R, Sari M A, and J T Ross, 1991, Expression of mammalian cytochrome P450 enzymes using yeast-based vectors *Methods Enzymol*, 206, 130.

Jefcoate C R, 1979, Measurement of substrate and inhibitor binding
25 to microsomal cytochrome P450 by optical difference spectroscopy. *Methods Enzymol*, 52, 258.

Lowry O H, Rosebrough N J, Farr L and Randali R J, 1951, Protein measurement with the Folin phenol reagent. *J. Biol. Chem*, 31,
30 4515.

Mehmood Z, Kelly D E, and S L Kelly, 1995, Metabolism of the herbicide chlortoluron by human cytochrome P450 3A4. *Chemosphere*, 31, 4515.

- 5 Omura T and R J Sato, 1964, The carbon-monoxide pigment of liver microsomes I. Evidence for its haemoprotein nature. *J. Biol. Chem.* 239, 2370.

10 Using the above methods the inventors have shown that against cyclosporin as a substrate and monitoring 3 different metabolites produced by human cytochrome P450 3A4, the IC_{50} values (the concentration required to reduce enzymic activity by 50%) are less than 140 μ M [micromolar] with nootkatone. Nootkatone has a high affinity for the cytochrome P450 enzyme with a K_s of 80-100 μ M.

15

In similar experiments using nootkatol a less marked inhibitory effect was observed.

20 **Example 2 - Effect of nootkatone on Taxol metabolism by human cytochrome P450 2C8 and human cytochrome P450 3A4 enzymes**

The Figure 1 graph of the effects of nootkatone on the formation of Taxol metabolites gives results of *in vitro* assays for the inhibition of the conversion of Taxol to 6-hydroxytaxol by human P450 2C8 and the
25 conversion of Taxol to "metabolite B" by human P450 3A4.

It is expected and observed by skilled persons that measurements of *in vitro* inhibition of these oxidative enzyme systems are indicative of *in vivo* activities. Thus, for example, literature reference to studies on
30 cytochrome P450 inhibitors (Clin. Pharmacol. Ther., 1991, 50, 394) also

quotes supporting references to parallel effects measured for the metabolism of drugs shown *in vivo* being seen as *in vitro*.

Use of a sesquiterpene in the inhibition of an oxidative enzyme

5

The inhibition of an oxidative enzyme can be readily determined by a skilled person in an *in vitro* test in which the consumption of oxygen by the oxidative enzyme is measured in the presence or absence of a sesquiterpene of the invention. The total inhibition or reduction of oxygen consumption in the presence of a sesquiterpene is indicative of an inhibitory effect of the invention.

Example 3 - *IN VIVO* INHIBITION TEST

15

The *in vitro* effect of the inhibitory sesquiterpenes of the invention on the metabolism of drugs exhibiting instability in the presence of an oxidative enzyme can be confirmed *in vivo* using the methods described by Edgar *et al*, (1992) Eur. J. Clin. Pharmacol., 42: 313-317, the disclosure and supporting references of which are incorporated herein by reference.

20

Edgar *et al* (1992) examined the effect of grapefruit juice on the pharmacokinetics in humans of felodipine, but the procedures disclosed can be applied to any drug by a skilled person. Subjects are given a 5mg dose of the drug and either water (200ml) or a solution (7mg in 200ml water) of the sesquiterpene of the invention. Blood samples (5ml) are taken for drug and its primary metabolite analysis before and after dosing. Samples are centrifuged and the plasma fraction analysed by gas chromatography. The maximum plasma concentration C_{max} and the time taken to reach the maximum t_{max} are recorded from the individual plasma

25
30

concentration-time curves. The area under the plasma concentration-time curve from time zero to infinity is calculated.

A positive inhibitory effect on drug metabolism is indicated by an increase
5 in C_{\max} and AUC in association with sesquiterpene intake compared to the values obtained with the drug in association with water intake.

Example 4 - SELECTION METHOD

10 Detection of Cytochrome P450 Inhibitor Activity in a Whole Cell Assay

The suitability of test sesquiterpene compounds as useful inhibitors of cytochrome P450 enzyme activity can be determined by introducing the
15 test compound to a cell preparation of an organism which produces a relevant oxidative enzyme and to which is added a known substrate for the enzyme system. Thus the hydroxylation of a steroid, such as 4-androstene-3, 17-dione, by the oxidative enzyme system present in *Bacillus megaterium* ATCC 13368 can be used to detect relevant cytochrome P450
20 activity. Such cells are available from Schering Corporation, U.S.A. Using the conditions for growth, conversion and detection described in the literature [Dror and Freeman, *Appl. Environ. Microbiol.*, 1995, 61, 855] the test sesquiterpene inhibitor is added to the reaction mixture of grown cells of *Bacillus megaterium* prior to the addition of the steroid substrate
25 and the progress of the hydroxylation assayed chromatographically as described. Test compounds which reduce the extent of hydroxylation of the substrate at levels less than 500 μ g/ml are deemed to be suitable inhibitors for use in a method of manufacturing a medicament according to the invention. Preferably, an inhibitor will show activity at 100 μ g/ml
30 or less.

Example 5 - PHARMACEUTICAL FORMULATIONS

The following examples illustrate pharmaceutical formulations according to the invention in which the active ingredient is a drug which exhibits instability in the presence of an oxidative enzyme. The sesquiterpene component is preferably one or more of nootkatone, nootkatol and α -vetivone.

Example A: Tablet

10

Sesquiterpene	7 mg
Active ingredient	100 mg
Lactose	200 mg
Starch	50 mg
15 Polyvinylpyrrolidone	5 mg
Magnesium stearate	4 mg
	<hr/>
	356 mg

20 Tablets are prepared from the foregoing ingredients by wet granulation followed by compression.

Example B: Tablet Formulations

25 The following formulations A and B are prepared by wet granulation of the ingredients with a solution of povidone, followed by addition of magnesium stearate and compression.

Formulation A

	<u>mg/tablet</u>	<u>mg/tablet</u>
(i) Sesquiterpene	7	7
(a) Active ingredient	250	250
5 (b) Lactose B.P.	210	26
(c) Povidone B.P.	15	9
(d) Sodium Starch Glycolate	20	12
(e) Magnesium Stearate	5	3
	—	—
10	507	507

Formulation B

	<u>mg/tablet</u>	<u>mg/tablet</u>
(i) Sesquiterpene	15	15
15 (a) Active ingredient	250	250
(b) Lactose	150	-
(c) Avicel PH 101*	60	26
(d) Povidone B.P.	15	9
(e) Sodium Starch Glycolate	20	12
20 (f) Magnesium Stearate	5	3
	—	—
	515	315

Formulation C

	<u>mg/tablet</u>
Sesquiterpene	7
Active ingredient	100
5 Lactose	200
Starch	50
Povidone	5
Magnesium stearate	4
	—
10	366

The following formulations, D and E, are prepared by direct compression of the admixed ingredients. The lactose used in formulation E is of the direction compression type.

15

Formulation D

	<u>mg/capsule</u>
Sesquiterpene	15
Active Ingredient	250
20 Pregelatinised Starch NF15	150
	—
	415

Formulation E

	<u>mg/capsule</u>
Sesquiterpene	15
Active Ingredient	250
5 Lactose	150
Avicel *	100
	—
	515

10 Formulation F (Controlled Release Formulation)

The formulation is prepared by wet granulation of the ingredients (below) with a solution of povidone followed by the addition of magnesium stearate and compression.

15		<u>mg/tablet</u>
	(i) Sesquiterpene	30
	(a) Active Ingredient	500
	(b) Hydroxypropylmethylcellulose (Methocel K4M Premium)*	112
20	(c) Lactose B.P.	53
	(d) Povidone B.P.C.	28
	(e) Magnesium Stearate	7
		—
		730

25

Drug release takes place over a period of about 6-8 hours and is complete after 12 hours.

Example C: Capsule FormulationsFormulation A

- 5 A capsule formulation is prepared by admixing the ingredients of Formulation D in Example B above and filling into a two-part hard gelatin capsule. Formulation B (*infra*) is prepared in a similar manner.

Formulation B

10		<u>mg/capsule</u>
	(i) Sesquiterpene	15
	(a) Active ingredient	250
	(b) Lactose B.P.	143
	(c) Sodium Starch Glycolate	25
15	(d) Magnesium Stearate	2
		—
		435

Formulation C

20		<u>mg/capsule</u>
	(i) Sesquiterpene	15
	(a) Active ingredient	250
	(b) Macrogol 4000 BP	350
		—
25		665

Capsules are prepared by melting the Macrogol 4000 BP, dispersing the active ingredient and sesquiterpene in the melt and filling the melt into a two-part hard gelatin capsule.

Formulation D

	<u>mg/capsule</u>
Sesquiterpene	15
Active ingredient	250
5 Lecithin	100
Arachis Oil	100
	—
	465

- 10 Capsules are prepared by dispersing the active ingredient in the lecithin and arachis oil and filling the dispersion into soft, elastic gelatin capsules.

Formulation E (Controlled Release Capsule)

- 15 The following controlled release capsule formulation is prepared by extruding ingredients i, a, b, and c using an extruder, followed by spheronisation of the extrudate and drying. The dried pellets are then coated with release-controlling membrane (d) and filled into a two-piece, hard gelatin capsule.

	<u>mg/capsule</u>
(i) Sesquiterpene	15
(a) Active ingredient	250
(b) Microcrystalline Cellulose	125
(c) Lactose BP	125
25 (d) Ethyl Cellulose	13
	—
	528

Example D: Injectable Formulation

Sesquiterpene 0.01 g

Active ingredient 0.200 g

- 5 Sterile, pyrogen free phosphate buffer (pH7.0) to 10 ml

The active ingredient and terpene are dissolved in most of the phosphate buffer (35-40°C), then made up to volume and introduced into a sterile 10 ml amber glass vial (type 1) and sealed with sterile closures and overseals.

10

Example E: Intramuscular injection

Sesquiterpene 0.10 g

Active ingredient 0.20 g

- 15 Benzyl Alcohol 0.10 g

Glucofurol 75* 1.45 g

Water for Injection q.s. to 3.00 ml

The active ingredient and sesquiterpene are dissolved in the glycofurol.

- 20 The benzyl alcohol is then added and dissolved, and water added to 3 ml.
The mixture is then filtered through a sterile micropore filter and sealed in sterile 3 ml glass vials (type 1).

Example F: Syrup Suspension

	Sesquiterpene	0.0125 g
	Active ingredient	0.2500 g
5	Sorbitol Solution	1.5000 g
	Glycerol	2.0000 g
	Dispersible Cellulose	0.0750 g
	Sodium Benzoate	0.0050 g
	Flavour, Peach 17.42.3169	0.0125 ml
10	Purified Water q.s. to	5.0000 ml

The sodium benzoate is dissolved in a portion of the purified water and the sorbitol solution added. The terpene and active ingredient are added and dispersed. In the glycerol is dispersed the thickener (dispersible
 15 cellulose). The two dispersions are mixed and made up to the required volume with the purified water. Further thickening is achieved as required by extra shearing of the suspension.

Example G: Suppository

20		<u>mg/suppository</u>
	Sesquiterpene (63 μ m)*	15
	Active ingredient (63 μ m)*	250
	Hard Fat, BP (Witepsol H15 - Dynamit Nobel)	1770
		—
25		2035

*The sesquiterpene and active ingredient are used as a powder wherein at least 90% of the particles are of 63 μ m diameter or less.

30 One fifth of the Witepsol H15 is melted in a steam-jacketed pan at 45°C

maximum. The sesquiterpene and active ingredient are sifted through a 200 μm sieve and added to the molten base with mixing, using a silverson fitted with a cutting head, until a smooth dispersion is achieved. Maintaining the mixture at 45°C, the remaining Witepsol H15 is added to the suspension and stirred to ensure a homogenous mix. The entire suspension is passed through a 250 μm stainless steel screen and, with continuous stirring, is allowed to cool to 40°C. At a temperature of 38°C to 40°C 2.02 g of the mixture is filled into suitable plastic moulds. The suppositories are allowed to cool to room temperature.

10

Example H: Pessaries

	<u>mg/pessary</u>
Sesquiterpene	15
Active ingredient	250
15 Anhydrate Dextrose	380
Potato Starch	363
Magnesium Stearate	7
	<hr/>
	1015

20

The above ingredients are mixed directly and pessaries prepared by direct compression of the resulting mixture.

CLAIMS

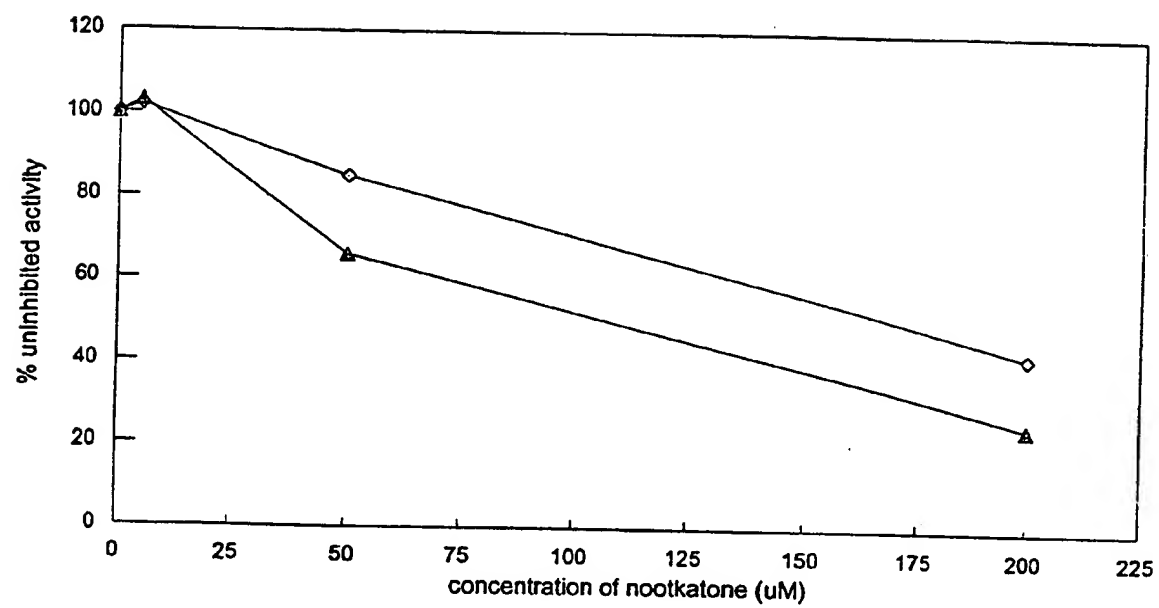
1. A sesquiterpene for use as a medicament.
- 5 2. Use of a sesquiterpene in the inhibition of one or more oxidative enzymes.
3. Use of a sesquiterpene in the manufacture of a composition for stabilising a drug compound *in vivo*, wherein the drug compound exhibits
10 instability in the presence of one or more oxidative enzymes.
4. Use of a sesquiterpene in the manufacture of a composition for stabilising a drug compound *in vitro*, wherein the drug compound exhibits instability in the presence of one or more oxidative enzymes.
15
5. A selection method comprising screening a test sesquiterpene compound for an inhibitory effect on the activity of an oxidative enzyme and, if the test compound displays an inhibitory effect, using the compound in the manufacture of a medicament as claimed in Claim 1.
20
6. A selection method as claimed in Claim 5, wherein the test compound is screened for an inhibitory effect on the oxidation of valencene to nootkatol.
- 25 7. A selection method as claimed in Claim 5, wherein the test compound is screened for an inhibitory effect on the hydroxylation of 4-androstene-3, 17-dione by *B megaterium* cells.
8. A sesquiterpene as claimed in any one of Claims 1 to 4 which is
30 bicyclic.

9. A sesquiterpene as claimed in any one of Claims 1 to 8 which is substituted.
10. A sesquiterpene as claimed in any one of Claims 1 to 9 which is
5 oxidised.
11. A sesquiterpene as claimed in Claim 10 which is nootkatone or an isomer thereof.
- 10 12. A pharmaceutical composition comprising a sesquiterpene and a pharmaceutically acceptable carrier.
13. A pharmaceutical composition as claimed in Claim 12 further comprising a drug which exhibits instability in the presence of an
15 oxidative enzyme.
14. A pharmaceutical composition as claimed in Claim 13 wherein the drug exhibits instability in the presence of a cytochrome P450 enzyme.
- 20 15. A pharmaceutical composition as claimed in Claim 14 wherein the cytochrome P450 enzyme is cytochrome P450 3A4.
16. A pharmaceutical composition as claimed in any one of Claims 13 to 15 wherein the drug is a dihydropyridine.
- 25 17. A pharmaceutical composition as claimed in any one of Claims 13 to 15 wherein the drug is cyclosporin.
18. A pharmaceutical composition as claimed in any one of Claims 13 to 15 wherein the drug is Taxol.
- 30

19. A pharmaceutical composition as claimed in any one of Claims 12 to 18 in the form of a solid.
20. A pharmaceutical composition as claimed in any one of Claims 12 to 18 in the form of a liquid.
21. A pharmaceutical composition as claimed in any one of Claims 12 to 20 adapted for oral administration.
22. A pharmaceutical composition as claimed in Claim 20 adapted for intravenous administration.
23. A pharmaceutical composition as claimed in any one of Claims 13 to 22 wherein the sesquiterpene is included in an amount of from 0.1 to 10% by weight based on the amount of drug in the composition.
24. A method of *in vitro* inhibition of an oxidative enzyme with a substituted or unsubstituted sesquiterpene comprising mixing the oxidative enzyme with the sesquiterpene.
25. A product comprising a sesquiterpene and a drug compound which exhibits instability in the presence of an oxidative enzyme as a preparation for simultaneous, separate or sequential use in therapy.

1/1

FIG. 1



—◇— human P450 3A4 enzyme
—△— human P450 2C8 enzyme